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Isolation and Characterization of PKC-L, A New Member of the Protein Kinase C-Related Gene Family Specifically Expressed in Lung, Skin, and Heart

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We have isolated and characterized a new human cDNA, coding for a protein kinase, related to the protein kinase C (PKC) gene family. Although this protein kinase shares some homologous sequences and structural features with the four members of the PKC family initially isolated (α, βI, βII, and γ), it shows more homology with the recently described PKC-related subfamily, encoded by the cDNAs δ, ε, and ζ. The transcript for this gene product, termed PKC-L, is most abundant in lung tissue, less expressed in heart and skin tissue, and exhibited very low expression in brain tissue. Thus, its tissue distribution is different from that described for other mammalian members of the PKC gene family, their expression being enriched in brain tissues. PKC-L is also expressed in several human cell lines, including the human epidermoid carcinoma line A431. The ability of phorbol esters to bind to and stimulate the kinase activity of PKC-L was revealed by introducing the cDNA into COS cells.

Protein kinase C (PKC) is involved in one of the major signal transduction systems, activated upon external stimulation of cells by various ligands including hormones, neurotransmitters, and growth factors (reviewed in references 25 and 26). These external signals induce the hydrolysis of membrane inositol phospholipids, generating, among other products, inositol triphosphate (IP_3) and diacylglycerol (DAG); IP_3, by mediating Ca^{2+} mobilization (4, 5), may act synergistically with DAG to activate PKC (17, 25, 40). Both IP_3 and DAG are rapidly degraded and exist only transiently after transmembrane signaling (4). Phorbol esters, which are capable of tumor promotion, can substitute for DAG in enzyme activation but, unlike diacylglycerol, are not rapidly metabolized (7). Activation of PKC, the high-affinity phorbol ester receptor (1, 7, 22, 24), is regarded as responsible, at least in part, for their tumor promotion activity.

Molecular cloning has revealed that PKC exists as a family of multiple subspecies having closely related structures (26). Four subspecies of this enzyme, α, βI, βII, and γ, were initially characterized (8, 16, 18, 28, 33, 34), and three were mapped to distinct chromosomal locations (8). Diversity within this family is further increased by alternative splicing (9, 32). These isoenzymes consist of a single polypeptide with four conserved (C_1 to C_4) domains separated by five variable (V_1 to V_5) regions. C_1 and C_2 are part of the regulatory domain, interacting with phospholipids and Ca^{2+}, while C_3 and C_4 are contained within the protein kinase domain. The C_1 region is characterized by a cysteine-rich repeat, which has been shown recently to be required for phorbol ester binding (29). These isoenzymes exhibit slightly different modes of activation, kinetic properties, and tissue distributions (19, 28, 44).

Recently, three additional cDNA clones, designated δ, ε, and ζ, were isolated from a rat brain cDNA library (30, 31). Other cDNA clones, nPKC and RP16, isolated from rabbit and rat brain cDNA libraries, encode the PKC-ε subspecies (16, 27). These three PKC-related molecules, δ, ε, and ζ, have structural features clearly distinct from those of the four subspecies described above. Most interestingly, all three subspecies lack the C_2 region, presumable involved in Ca^{2+} binding (27, 30, 31).

PKC species have also been isolated from *Drosophila melanogaster* (35, 38). Three isoenzymes have been characterized so far, including one specifically expressed in photoreceptor cells (38). The other two isoenzymes are most similar to PKC-α and PKC-δ (35, 38).

In this paper we describe the isolation of a new human protein kinase gene which is expressed predominantly in lung tissue and is therefore termed PKC-L. Our data suggest that PKC-L is a new member of the PKC-related subfamily. The cellular function and signaling pathways activated by these PKC-related protein kinases are presently unknown.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones. cDNA libraries prepared from human keratinocytes and human lung tissue (Clontech), constructed in λgt11, were used for the isolation of bacteriophage clones encoding PKC-L (see Fig. 1). A human keratinocyte cDNA library was initially screened by using two labeled cDNA fragments obtained from PKC-α as probes: human PKC-α (λhPKC-α7), nucleotides 1 to 519 (probe A); and bovine PKC-α (λbPKC-α306), nucleotides 952 to 2182 (probe B) (34). Each of the cDNA fragments was ^{32}P labeled by the random-priming procedure (13). One million plaques were hybridized first in duplicate filters with probe A. Selected plaques were hybridized alter with probe B. Hybridization was carried out at 65°C under standard conditions. Following hybridization, filters were washed under low-stringency conditions with 3× SSC–0.1% sodium dodecyl sulfale (SDS) at 50°C for 1 h (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.4]). High-stringency screening involved washing at 65°C in 0.1× SSC–0.1% SDS. Filters were exposed to X-ray films for 15 to 20 h at −70°C, with an intensifying screen. Plaques exhibiting positive signals with both probes under only low-stringency hybridization conditions were selected for further studies.

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The 1.8-kb insert, obtained from two independent phages, was subcloned into the *Eco*RI site of pUC18 plasmids. The nucleic acid sequence was determined by the strand-terminating method (37) on both strands, following subcloning of specific restriction fragments into M13mp18 or M13mp19. The modified T7 DNA polymerase-Sequenase (United States Biochemical Corp.) was used for both double-stranded and single-stranded sequencing; standard protocols were followed.

Cell culture. Human epidermal carcinoma A431 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. The human skin keratinocyte HaCaT cell line (6), and the human skin squamous carcinoma SCL-1 cell line (41) were kindly provided by N. E. Fusenig and grown in a 1:1 mixture of DMEM and F12 medium (GIBCO) containing 10% fetal calf serum.

Northern (RNA) blots. Total RNA was prepared from rat tissues by using the previously described urea-LiCl procedure (2). Poly(A)$^+$ RNA was further purified by oligo(dT)-cellulose column chromatography (3). Equal amounts of denatured RNA were fractionated on morpholinepropanesulfonic acid (MOPS)-formaldehyde-containing 1% agarose gels and transferred to nitrocellulose filters. Radioactive probes were prepared by hexanucleotide random priming and used for hybridization at 42°C in buffer consisting of 50% formamide, 5× SSC, 100 μg of sheared salmon sperm DNA per ml, 5x Denhardt solution (1x Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin [BSA]), and 50 mM sodium phosphate (pH 6.8). Following 24 h of hybridization, filters were washed for 2 h in several changes of 0.2× SSC–0.1% SDS at 45 or 65°C, dried, and exposed to an X-ray film for 2 days at −70°C with an intensifying screen.

Polymerase chain reaction. cDNA for use in the polymerase chain reaction was prepared by using poly(A)$^+$ mRNA from the human skin carcinoma SCL-1 cell line (41) as described above. Oligo(dT)$_{18}$ primer was annealed to 1 μg of mRNA in 15 μl of annealing mixture containing 0.83 μM primer, 55 mM Tris hydrochloride (pH 8.3), and 40 mM KCl. cDNA was synthesized by the addition of 3.75 μl containing the four deoxynucleoside triphosphates (4 mM each), 33 mM $MgCl_2$, 0.1 μl of RNasin (Promega), 17 mM dithiothreitol, and 1 μl of reverse transcriptase (super RT; Molecular Genetic Resources). Following incubation for 1 h at 41°C, this reaction mixture was used directly in the polymerase chain reaction, essentially as described previously (36). The final volume of 100 μl contained, in addition to the cDNA reaction mixture, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris hydrochloride (pH 8.8), 6.7 mM $MgCl_2$, 6.7 μM EDTA, 0.17 of BSA per ml, 10% dimethyl sulfoxide, 0.5 μM "forward" oligonucleotide primer (Fig. 1, nucleotides 1737 to 1759), 0.5 μM oligo(dT)$_{18}$ primer, the four deoxynucleoside triphosphates (1 mM each), and 10 mM dithiothreitol. This mixture was boiled for 7 min and cooled on ice, and 2 U of Taq polymerase and 100 μl of mineral oil were added. This mixture was subjected to 35 cycles of annealing (2 min at 56°C), elongation (10 min at 72°C), and denaturation (1 min at 95°C). Amplified fragments were isolated from agarose gels and cloned into the *Sam*I site of Bluescript KS$^+$ (Stratagene).

Construction of expression plasmids. The 1.8-kb *Eco*RI DNA fragment from λM2 was cloned into the *Xho*I site of the simian virus 40-based expression vector pX3, previously described (23), by using *Xho*I DNA linkers. The resultant plasmid obtained was denoted pXM2. The plasmid pXKF contained the full-length cDNA coding for bovine PKC-α, subcloned into the same vector by using similar procedures. The full-length cDNA of PKC-L was constructed in pUC18 as follows. λM2 subcloned in pUC18 was digested with *Hinc*II and *Bgl*II and with *Bgl*II and *Pst*I. The fragments isolated, of 4.2 kb (including the pUC18 vector) and 0.17 kb, respectively, were ligated in a three-part ligation mixture with a 0.6-kb fragment isolated from λL17 by digestion with *Pst*I and *Eco*RV. Correct assembly of cDNA fragments was also verified by DNA sequencing. The full-length PKC-L in pUC18 was digested by *Eco*RI and *Hin*dIII, and the staggered ends were filled in with the Klenow fragment of DNA polymerase I. The blunt-ended fragment was ligated into the *Sam*I site of pLSV-derived pX3 vector (23).

Transfection to COS cells. Transfection experiments were performed by the DEAE-dextran procedure (39) with some modifications. COS cells from a confluent tissue culture dish were split 1:5 2 days before transfection. Cells were carefully washed with DMEM and incubated with 5 ml of DMEM containing 50 mM Tris hydrochloride (pH 7.3), 0.2 mg of DEAE-dextran (molecular weight, 2 × 10^6; Pharmica) per ml, and 10 μg of plasmid DNA. At 6 to 8 h later, cells were washed extensively and DMEM containing 10% fetal calf serum was added. The cells were assayed after additional 48 to 72 h of incubation.

Phorbol ester binding assay. Transfected COS cells were washed and incubated for 1 h at 37°C with binding buffer (DMEM containing 1% BSA and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5]) 48 to 72 h after transfection. Following incubation, this medium was replaced by 2.5 ml of binding buffer containing 5 nM [^{3}H]PDBU ([^{3}H]phorbol-12,13-dibutyrate; Amersham), and incubation was continued for 30 min at 37°C. After being washed three times with binding buffer, cells were solubilized in 1 ml of 10% SDS and the radioactivity was measured. Nonspecific binding was determined by parallel incubation of transfected dishes with 5 μM unlabeled PDBU.

Partial purification of PKC-L. COS cells transfected with various plasmids were disrupted in homogenization buffer exactly as described previously (11). Following centrifugation, the supernatant was applied onto a 0.5-ml DEAE-Sephacel column and eluted with 0.3 M NaCl in column buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10% glycerol, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of leupeptin per ml. DEAE-eluted fractions (250 μl) were incubated for 120 min at 4°C with anti–PKC-L antibody (no. 241) bound to Sepharose-protein A (Pharmacia). Immunoprecipitates were washed three times with washing buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Antibody no. 241 is a polyclonal antibody raised against a 14-amino-acid peptide (amino acids 300 to 313) deduced from the sequence of PKC-L (3a).

Phosphorylation experiments. The kinase activity of immune complexes was determined shortly after partial purification of COS cell lysates. The reaction mixture for autophosphorylation (20 μl) contained 50 mM HEPES (pH 7.5), 15 mM magnesium acetate, 1 μM ATP, 1 mM $CaCl_2$, 50 μg of L-α-phosphatidylserine per ml, 1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and 5 μCi of [γ-^{32}P]ATP. Ca^{2+} or phosphatidylserine and TPA were omitted from the reaction when indicated. The reaction was carried out at 20°C for 15 min. Sample buffer was added to stop the reaction, and the samples were heated at 95°C for 3 min before being loaded onto a SDS–7.5% acrylamide gel.

Nucleotide sequence accession number. The nucleotide sequence discussed in this paper has been assigned the GenBank accession number M55284.

RESULTS

Isolation of PKC-related cDNA clones. The cDNA described above was initially isolated by screening a human keratinocyte cDNA library at reduced stringency with a probe derived from hPKC-α (8). Among several of the identified PKC-related cDNA clones, λM2 was further characterized (Fig. 1A). λM2, spanning 1,831 bp, contains an open reading frame of 1,730 nucleotides with a potential ATG initiation codon at nucleotides 167 to 169. This ATG is flanked by sequences that fulfil the Kozak criteria for initiation codons (20, 21) and lies within a G+C-rich nucleotide sequence (>80% G+C). The long open reading frame is preceded by an in-frame termination codon at nucleotides 101 to 103. It should be noted however, that an additional potential initiation codon occurs in a right nucleotide context at nucleotides 182 to 185, just downstream of the predicted initiation codon. The scanning model for initiation of translation (20, 21) predicts that proximity to the capped 5′ end of the mRNA determines the ATG actually functioning in initiation. However, we cannot rule out the possibility that in this particular mRNA, "leaky scanning" may dictate translation from the second AUG also.

Our attempts to isolate the 3′ end of the cDNA from the human keratinocyte cDNA library by using a probe derived from λM2 (*Bam*HI-*Eco*RI) were not successful. It is possible that this region was underrepresented in the keratinocyte cDNA library for reasons that we do not understand. Therefore, to isolate the 3′ end of the coding region, we generated a DNA probe by using the polymerase chain reaction. Oligo(dT)$_{18}$ and a synthetic oglionucleotide corresponding to nucleotides 1755 to 1776 of the coding strand of λM2 amplified a ~0.45-kb fragment, PC5 (Fig. 1A), by using mRNA prepared from a human skin carcinoma cell line (41). The nucleotide sequence corresponding to its 5′ end overlapped exactly with the sequence of λM2. However, PC5 did not contain poly(A)-like sequences at its 3′ end. Apparently, under the reaction condition used, the same synthetic primer (22-mer) was also used for amplification at the 3′ end, by annealing to a related nonidentical sequence with several mismatches (nucleotides 2197 to 2217). The amplified fragment was used as a probe to isolate the overlapping cDNA clone λL17 from a human lung cDNA library (Fig. 1A). The nucleotide sequence obtained from λL17 is shown in Fig. 1B and corresponds to nucleotides 1595 to 2382. This sequence overlapped exactly with nucleotides 1595 to 1831 of λM2 and with the nucleotide sequence of PC5 (nucleotides 1755 to 2201). Although λM2 and λL17 were isolated from different human tissues, the identity of 236 bases between these clones (or of 446 bases between PC5 and λL17) suggests that they were derived from a single mRNA.

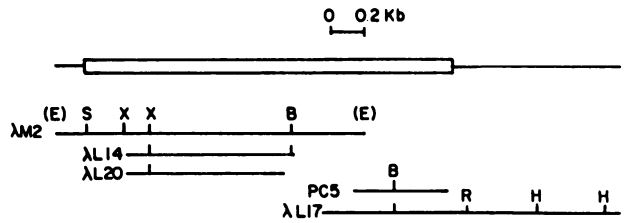
Sequence comparisons with other PKC genes. Alignment of deduced amino acids encoded by the composite cDNA of PKC-L with other members of the PKC gene family is shown in Fig. 2. PKC-L shares greater homology and a similar overall structure with members of the recently identified PKC-related subfamily PKC-δ, PKC-ε, and PKC-ζ, (16, 28, 30, 31) than with the classical PKC-α, PKC-β, and PKC-γ (8, 16, 18, 28, 33, 34). Among members of this subfamily, PKC-L exhibits the highest homology with PKC-ε (16, 27, 30). Homology with PKC subspecies is greatest at the carboxy-terminal half, which contains the consensus ATP-binding site GXGXXGX$_{16}$K and the catalytic kinase domain (Fig. 2A). At this kinase region, PKC-L shares 59% homology with the α and β forms, 54% homology with the γ form, and 70, 53, and 47% homology with the ε, δ, and ζ forms, respectively.

The regulatory domain of PKC molecules contains a highly conserved cysteine-rich domain. This cysteine-rich zinc finger motif has also been found in many nucleic acid-binding proteins (12). Cysteine-rich sequences appear as a tandem repeat in most PKC subspecies (except PKC-ζ [31]). Alignment of their sequences with amino acid sequences deduced from PKC-L revealed 40 to 42% homology with PKC-α, PKC-β, or PKC-γ and 65, 36, and 37% homology with PKC-ε, PKC-δ, and PKC-ζ, respectively. All six cysteines contained in the first cysteine-rich repeat were fully conserved in PKC-L, whereas two of the six conserved cysteines were missing at the second cysteine repeat (Fig. 2B). Recent studies with PKC-γ have indicated that this cysteine-rich zinc finger-like domain was essential for phorbol ester binding (29).

Another structural feature shared by all classical PKC molecule is the pseudosubstrate sequence (15) located at their N terminus. This 8-amino-acid sequence X-X-Gly-Ala-Leu-X-X-X (where X is a basic residue), resembling the PKC phosphorylation site on target proteins, was implicated in the regulation of the kinase activity of the enzyme (15). In PKC-ε and PKC-δ this sequence has slightly diverged, although basic amino acids are present at similar positions. In PKC-L it has diverged even further (Fig. 2B); therefore, its ability to serve as an inhibitor for kinase activity has yet to be examined. Diverged sequences may reflect differences in substrate specificities for each of these individual kinases. It should be noted, however, that homology of PKC-L with other PKC-related family members is considerably reduced beyond the kinase and the cysteine-rich domains. The sequence separating these two domains (amino acids 307 to 343) is variable in sequence and length among all PKC-related molecules. At the N-terminal region (amino acids 1 to 152) of the molecule, PKC-L shares only 50, 21, and 15% homology with PKC-ε, PKC-δ, and PKC-ζ, respectively (an analogous region is lacking in PKC-α, PKC-β, and PKC-γ).

Tissue-specific expression. Northern blot analysis of rat mRNA isolated from various tissues, probed by cDNA fragments coding for PKC-L, identified a single mRNA transcript ~4.2 kb long (Fig. 3). Expression of this mRNA displayed tissue-specific distribution. It was most abundant in lung tissues, was present at lower levels in heart and skin tissues, and was present at very low levels (or absent) in brain tissues (Fig. 3). A cDNA fragment (*Eco*RI-*Xho*I of λM2; 410 bp) derived from the variable region at the 5′-terminal end of the gene (Fig. 1A) was used as the probe in this analysis. Exactly the same tissue distribution was found when two other cDNA fragments derived from the kinase domain (*Bam*HI-*Eco*RI of λM2, 437 bp) and from the 3′ end of λL17 (*Bam*HI-*Eco*RI of λL17; 1,460 bp) were used as probes (data not shown). These data indicated again that λM2 and λL17 originated from the same mRNA. Although PKC-L exhibits homology with PKC-ε at specific regions (Fig. 2), their different tissue distributions and transcript sizes rule out the possibility that PKC-L represents the human homolog of rat PKC-ε (or rabbit nPKC). The single mRNA transcript detected by PKC-ε cDNA probes in rat tissues was 7.1 kb long and was expressed mainly in brain tissue (30). Another cDNA clone, RP16, which had an almost identical sequence to PKC-ε, was isolated by Housey et al. (16) from a rat brain cDNA library. Similarly, RP16

A



B

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1    GAATTCGGACGGAGGAGGCAGAATGGCCAGTCGAGGGGCGCTTAGGCGTGGCCTTTCCCCAGGGCTGCCTCGACTCCTGCACCTGTCCCGAGGGCTGG   98
99   CCTGAGACGGGACTCCCGGTTCTCCCGCTGCGAAGCAGGCCCCCCGGGGCCGGGGCAGCGGCGCCGGCATGTCGTCTGGCACCATGAAGTTCAATGGC  196
                                                                  *  M  S  S  G  T  M  K  F  N  G   10

197  TATTTGAGGGTCCGCATCGGTGAGGCAGTGGGGCTGCAGCCCACCCGCTGGTCCCTGCGCCACTCGCTCTTCAAGAAGGGCCACCAGCTGCTGGACCC  294
     Y  L  R  V  R  I  G  E  A  V  G  L  Q  P  T  R  W  S  L  R  H  S  L  F  K  K  G  H  Q  L  L  D  P   43

295  CTATCTGACGGTGAGCGTGGACCAGGTGCGCGTGGGCCAGACCAGCACCAAGCAGAAGACCAACAAACCCACGTACAACGAGGAGTTTTGCGCTAACG  392
     Y  L  T  V  S  V  D  Q  V  R  V  G  Q  T  S  T  K  Q  K  T  N  K  P  T  Y  N  E  E  F  C  A  N  V   76

393  TCACCGACGGCGGCCACCTCGAGTTGGCCGTCTTCCACGAGACCCCCCTGGGCTACGACTTCGTGGCCAACTGCACCCTGCAGTTCCAGGAGCTCGTC  490
       T  D  G  G  H  L  E  L  A  V  F  H  E  T  P  L  G  Y  D  F  V  A  N  C  T  L  Q  F  Q  E  L  V  108

491  GGCACGACCGGCGCCTCGGACACCTTCGAGGGTTGGGTGGATCTCGAGCCAGAGGGGAAAGTATTTGTGGTAATAACCCTTACCGGGAGTTTCACTGA  588
     G  T  T  G  A  S  D  T  F  E  G  W  V  D  L  E  P  E  G  K  V  F  V  V  I  T  L  T  G  S  F  T  E  141

589  AGCTACTCTCCAGAGAGACCGGATCTTCAAACATTTTACCAGGAAGCGCCAAAGGGCTATGCGAAGGCGAGTCCACCAGATCAATGGACACAAGTTCA  686
       A  T  L  Q  R  D  R  I  F  K  H  F  T  R  K  R  Q  R  A  M  R  R  R  V  H  Q  I  N  G  H  K  F  M  174

687  TGGCCACGTATCTGAGGCAGCCCACCTACTGCTCTCACTGCAGGGAGTTTATCTGGGGAGTGTTTGGGAAACAGGGTTATCAGTGCCAAGTGTGCACC  784
       A  T  Y  L  R  Q  P  T  Y  C  S  H  C  R  E  F  I  W  G  V  F  G  K  Q  G  Y  Q  C  Q  V  C  T  206

785  TGTGTCGTCCATAAACGCTGCCATCATCTAATTGTTACAGCCTGTACTTGCCAAAACAATATTAACAAAGTGGATTCAAAGATTGCAGAACAGAGGTT  882
     C  V  V  H  K  R  C  H  H  L  I  V  T  A  C  T  C  Q  N  N  I  N  K  V  D  S  K  I  A  E  Q  R  F  239

883  CGGGATCAACATCCCACACAAGTTCAGCATCCACAACTACAAAGTGCCAACATTCTGCGATCACTGTGGCTCACTGCTCTGGGGAATAATGCGACAAG  980
       G  I  N  I  P  H  K  F  S  I  H  N  Y  K  V  P  T  F  C  D  H  C  G  S  L  L  W  G  I  M  R  Q  G  272

981  GACTTCAGTGTAAATATGTAAATGAATGTGCATATTCGATGTCAAGCGAACGTGGCCCTAACTGTGGGGTAATGCGGTGGAACTTGCCAAGACCTGGC  1078
       L  Q  C  K  Y  V  N  E  C  A  Y  S  M  S  S  E  R  G  P  N  C  G  V  M  R  W  N  L  P  R  P  G  304

1079 AGGGATGGTCTCCAACCCGGAAATATTTCTCCAACCTCGAAACTCGTTTCCAGATCGACCCTAAGACGACAGGGAAAGGAGAGCAGCAAAGAAGGAAA  1176
     R  D  G  L  Q  P  G  N  I  S  P  T  S  K  L  V  S  R  S  T  L  R  R  Q  G  K  E  S  S  K  E  G  N  337

1177 TGGGATTGGGGTTAATTCTTCCAACCGACTTGGTATCGACAACTTTGAGTTCATCCGAGTGTTGGGGAAGGGGAGTTTTGGGAAGGTGATGCTTGCAA  1274
     G  I  G  V  N  S  S  N  R  L  G  I  D  N  F  E  F  I  R  V  L  G  K  G  S  F  G  K  V  M  L  A  R  370
                                                                  ●     ●     ●

1275 GAGTAAAAGAAACAGGAGACCTCTATGCTGTGAAGGTGCTGAAGAAGGACGTGATTCTGCTGGATGATGATGTGGAATGCACCATGACCGAGAAAAGG  1372
       V  K  E  T  G  D  L  Y  A  V  K  V  L  K  K  D  V  I  L  L  D  D  D  V  E  C  T  M  T  E  K  R  402
                                    ▲

1373 ATCCTGTCTCTGGCCCGCAATCACCCCTTCCTCACTCAGTTGTTCTGCTGCTTTCAGACCCCCGATCGTCTGTTTTTTGTGATGGAGTTTGTGAATGG  1470
     I  L  S  L  A  R  N  H  P  F  L  T  Q  L  F  C  C  F  Q  T  P  D  R  L  F  F  V  M  E  F  V  N  G  435

1471 GGGTGACTTGATGTTCCACATTCAGAAGTCTCGTCGTTTTGATGAAGCACGAGCTCGCTTCTATGCTGCAGAAATCATTTCGGCTCTCATGTTCCTCC  1568
     G  D  L  M  F  H  I  Q  K  S  R  R  F  D  E  A  R  A  R  F  Y  A  A  E  I  I  S  A  L  M  F  L  H  468

1569 ATGATAAAGGAATCATCTATAGAGATCTGAAACTGGACAATGTCCTGTTGGACCACGAGGGTCACTGTAAACTGGCAGACTTCGGAATGTGCAAGGAG  1666
       D  K  G  I  I  Y  R  D  L  K  L  D  N  V  L  L  D  H  E  G  H  C  K  L  A  D  F  G  M  C  K  E  500

1667 GGGATTTGCAATGGTGTCACCACGGCCACATTCTGTGGCACGCCAGACTATATCGCTCCAGAGATCCTCCAGGAAATGCTGTACGGGCCTGCAGTAGA  1764
     G  I  C  N  G  V  T  T  A  T  F  C  G  T  P  D  Y  I  A  P  E  I  L  Q  E  M  L  Y  G  P  A  V  D  533

1765 CTGGTGGGCAATGGGCGTGTTGCTCTATGAGATGCTCTGTGGTCACGCGCCTTTTGAGGCAGAGAATGAAGATGACCTCTTTGAGGCCATACTGAATG  1862
     W  W  A  M  G  V  L  L  Y  E  M  L  C  G  H  A  P  F  E  A  E  N  E  D  D  L  F  E  A  I  L  N  D  566

1863 ATGAGGTGGTCTACCCTACCTGGCTCCATGAAGATGCCACAGGGATCCTAAAATCTTTCATGACCAAGAACCCCACCATGCGCTTGGGCAGCCTGACT  1960
       E  V  V  Y  P  T  W  L  H  E  D  A  T  G  I  L  K  S  F  M  T  K  N  P  T  M  R  L  G  S  L  T  598

1961 CAGGGAGGCGAGCACGCCATCTTGAGACATCCTTTTTTTAAGGAAATCGACTGGGCCCAGCTGAACCATCGCCAAATAGAACCGCCTTTCAGACCCAG  2058
     Q  G  G  E  H  A  I  L  R  H  P  F  F  K  E  I  D  W  A  Q  L  N  H  R  Q  I  E  P  P  F  R  P  R  631

2059 AATCAAATCCCGAGAAGATGTCAGTAATTTTGACCCTGACTTCATAAAGGAAGAGCCAGTTTTAACTCCAATTGATGAGGGACATCTTCCAATGATTA  2156
       I  K  S  R  E  D  V  S  N  F  D  P  D  F  I  K  E  E  P  V  L  T  P  I  D  E  G  H  L  P  M  I  N  664

2157 ACCAGGATGAGTTTAGAAACTTTTCCTATGTGTCTCCAGAATTGCAACCATAGCCTTATGGGGAGTGAGAGAGAGGGCACGAGAACCCAAAGGAATAG  2254
       Q  D  E  F  R  N  F  S  Y  V  S  P  E  L  Q  P  *                                                680

2255 AGATTCTCCAGGAATTTCCTCTATCGGACCTTCCCAGCATCAGCCTTAGAACAAGAACCTACCTTCAAGGAGCAAGTGAAGAACTCTGTCGAAGGATG  2352

2353 GAACTTTCAGATATCAACTATTTAGAGTCC  2382
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FIG. 1. Restriction maps and nucleic acid sequence of PKC-L clones. (A) Schematic presentation of the cDNA structure coding for PKC-L and its corresponding cDNA clones. Symbols: ——, untranslated sequence; ▭, coding sequence. Restriction enzymes used to construct this map were *Eco*RI (E), *Xho*I (X), *Bam*HI (B), *Sma*I (S), *Eco*RV (R), and *Hin*dIII (H). (B) Nucleotide sequence and deduced amino acid sequence of the PKC-L gene. The amino acid at the putative ATG initiation codon is numbered 1. Termination of the coding region and the first upstream in-frame stop codon at the 5′-untranslated sequence are indicated by asterisks. Glycine and lysine residues at the ATP-binding site are shown by dots and an arrow, respectively.

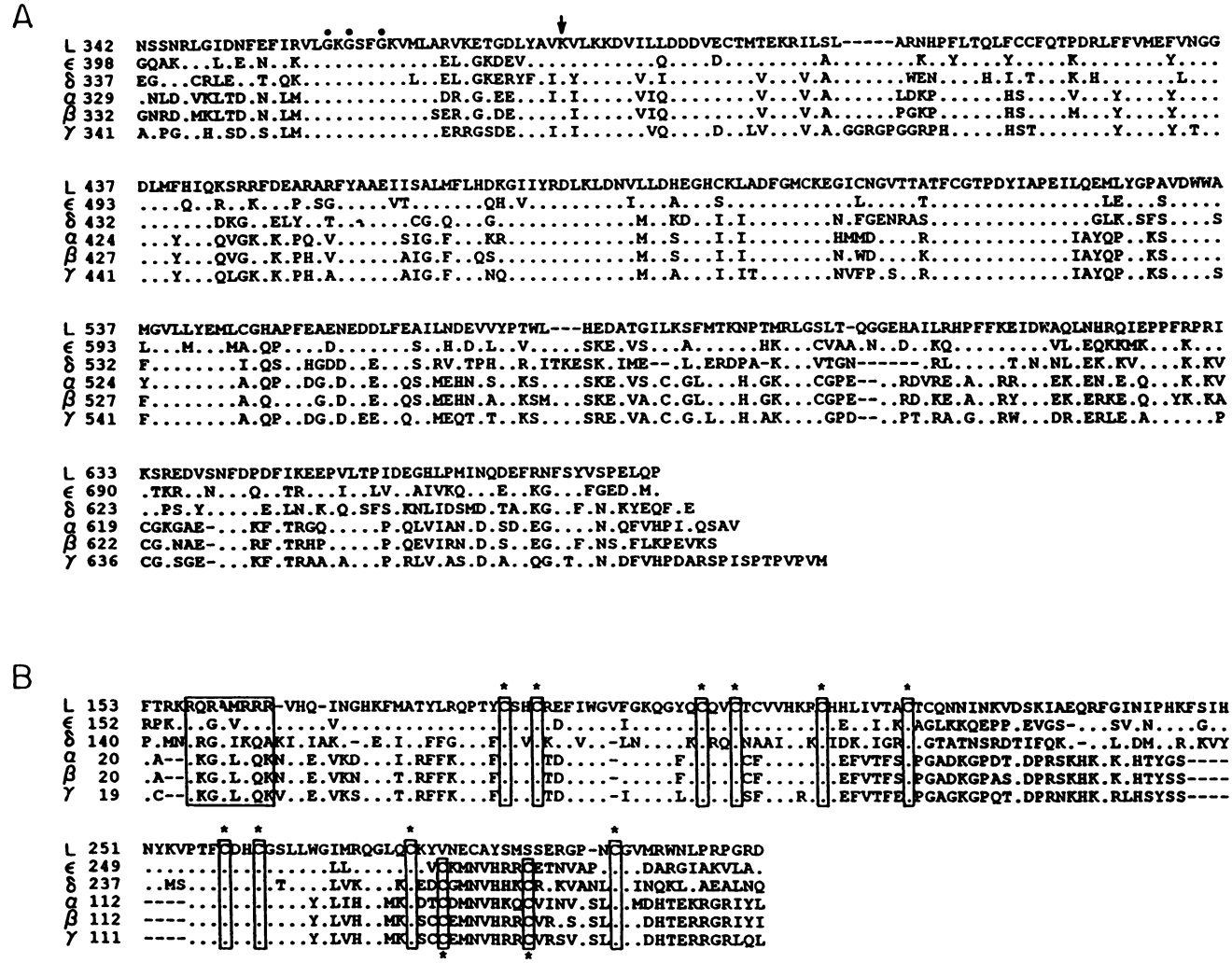

FIG. 2. Comparison of PKC-L sequences with the different PKCs. (A) Sequence alignment of kinase regions of the different PKC members. Sequences used for the alignment are bovine PKC-α (34), bovine PKC-β (8), bovine PKC-γ (8), rat PKC-ε (30), and rat PKC-δ (30). Dots and the arrow above the sequence indicate glycine and lysine residues constituting the ATP-binding site. Gaps were introduced to optimize alignment. Dots along the sequences represent identical residues. (B) Sequence alignment of the cysteine-rich repeat of the different PKC members. Sequences used for alignment are human PKC-α (34), human PKC-β (8), human PKC-γ (8), rat PKC-ε (30), and rat PKC-δ (30). Conserved cysteine residues and the putative pseudosubstrate sequences are boxed.

cDNA detected a single transcript of ca. 7.5 kb, highly expressed in brain tissues, at moderate levels in heart tissues, and at low levels in liver tissues (16). However, the mRNA transcript detected by PKC-L probes in rat tissues was only ~4.2 kb, exhibited very low expression, if any, in brain tissues, and was expressed mainly in lung tissues but also in skin and heart tissues (Fig. 3).

This tissue distribution led us to examine the expression of PKC-L in human skin-derived cell lines. Northern blot analysis revealed that the human mRNA coding for PKC-L, isolated from the human skin carcinoma line SCL-1 (41), is similar in size to rat mRNA isolated from skin tissues (Fig. 4A). The PKC-L mRNA transcript is expressed at similar levels in the human skin carcinoma line SCL-1 and the human skin keratinocyte HaCaT line. Most interestingly, the PKC-L transcript is also found in the well-studied human epidermoid carcinoma line A431 (Fig. 4B). For these cell lines the single mRNA transcript of 4.2 kb was exhibited with any of the cDNA fragments used, derived either from

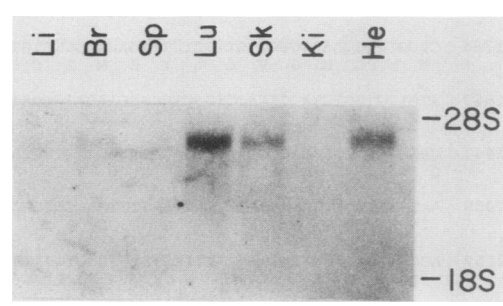

FIG. 3. Northern blot analysis of PKC-L gene expression. Equal amounts of poly(A)$^+$ RNA, extracted from various rat tissues, were fractionated on a 1% agarose–formaldehyde gel, blotted, and hybridized to a ^{32}P-labeled 0.41-kb DNA probe (*Eco*RI-*Xho*I fragment) derived from the 5′ region of the isolated cDNA of PKC-L. RNA shown was from liver (lane Li), brain (lane Br), spleen (lane Sp), lung (lane Lu), skin (lane Sk), kidney (lane Ki), and heart (lane He). The positions of 28S and 18S rRNAs are indicated as standards.

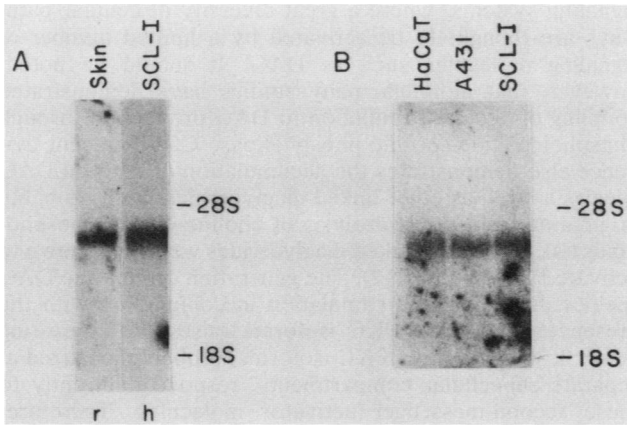

FIG. 4. Northern blot analysis of PKC-L gene expression in human cell lines. Equal amount of poly(A)$^+$ RNA, extracted from the indicated human cell lines, were fractionated on a 1% agarose–formaldehyde gel, blotted, and hybridized to ^{32}P-labeled DNA probes. (A) Comparison of the hybridization analysis of rat skin (lane r) and human SCL-1 (lane h) mRNAs. (B) Hybridization analysis of human HaCaT, A431, and SCL-1 mRNAs. The positions of 28S and 18S rRNAs are indicated as standards. The cDNA fragment *Eco*RI-*Xho*I (0.41 kb) was used for hybridiation in panel A, and the *Hin*dIII cDNA fragment (0.45 kb) was used in panel B.

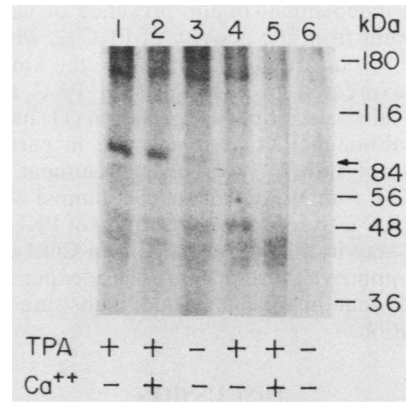

FIG. 6. Analysis of kinase activity of PKC-L expressed in COS cells. Transfected cells were solubilized, partially purified on DEAE-Sephacel columns, and immunoprecipitated with PKC-L-specific antibodies. Immunoprecipitates were incubated in an assay mixture (described in Materials and Methods) in the presence of [γ-^{32}P]ATP, with or without phorbol esters (TPA) and Ca^{2+} as indicated. The phosphorylated products were separated on SDS–7.5% polyacrylamide and then analyzed by autoradiography. Lanes 1 to 3 represent the immunoprecipitates from COS cells transfected by the full-length PKC-L-containing plasmid (pL10), and lanes 4 to 6 represent immunoprecipitates from COS cells transfected by control plasmid (pX3).

the 5′ end of the cDNA (*Eco*RI-*Xho*I fragment, exemplified for SCL-1 cells; Fig. 4A) or from the 3′ end of the cDNA (a *Hin*dIII fragment from the 3′-noncoding region; Fig. 4B).

Phorbol ester binding. To examine the ability of PKC-L gene product to bind phorbol esters, we have introduced its cDNA constructs into COS cells. Transfected COS cells were analyzed for the expression of high-affinity phorbol ester receptor activity. COS cells transfected with pXM2 plasmid, containing the cDNA construct of PKC-L, resulted in more than a fourfold increase in [^{3}H]PDBU-binding activity (Fig. 5). Binding assays were performed in the presence of 5 nM [^{3}H]PDBU, indicating that phorbol ester binding in these transfected cells represented high-affinity receptors. Introduction of the pXKF construct, containing the cDNA coding for PKC-α, into COS cells also conferred increased [^{3}H]PDBU-binding activity, similar to [^{3}H]PDBU-binding activity exhibited by PKC-L-transfected cells (Fig. 5), suggesting that the ability of PKC-L to bind phorbol ester is similar to that of PKC-α. COS cells express some endogenous PKC-α, which is probably responsible for the specific PDBU binding in control experiments.

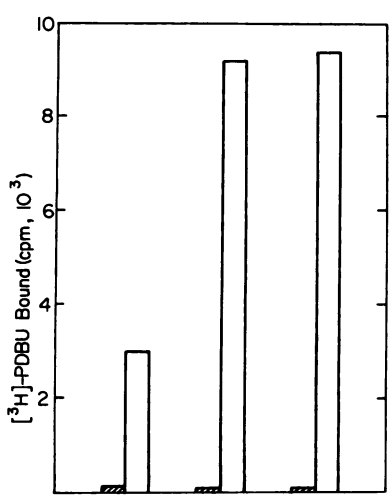

FIG. 5. Phorbol ester-binding activity of transfected COS cells. Each 10-cm dish of COS cells was transfected with to μg of the following plasmids: pXM2, coding for PKC-L; pXKF, coding for PKC-α; and the control plasmid pX3, lacking any cDNA insert. Three days after transfection, cells were subjected to a [^{3}H]PDBU binding assay in the presence (▨) or absence (□) of unlabeled PDBU (5 μM), as described in Materials and Methods. Background [^{3}H]PDBU binding activity originates from low endogenous PKC-α activity.

It has been shown recently that the conserved cysteine-rich zinc finger sequences are indispensable for binding phorbol esters (29). Either one of the two cysteine repeats contained in this domain appeared to be sufficient for phorbol esters binding, whereas mutation of conserved cysteines abolished binding (29). Therefore, it was interesting that PKC-ζ, having only one set of cysteine-rich sequences, failed to bind PDBU (31) upon transfection of its cDNA into COS cells. However, PKC-L, having one conserved repeat of cysteines but missing two cysteines at the second repeat (Fig. 2B), was still able to bind phorbol esters efficiently.

Kinase activity of PKC-L. We have analyzed the kinase activity of PKC-L to demonstrate that its direct interaction with phorbol esters can also regulate this intrinsic activity. PKC-L was immunoprecipitated (by using specific antibody no. 241) from COS cells transfected with the full-length cDNA-containing plasmid (pL10) and with control plasmids (pX3). Autophosphorylation of PKC-L was examined under conditions similar to those used for the phosphorylation of exogenously added substrates (11). PKC-L appeared as an 86-kDa protein on SDS-polyacrylamide gels (Fig. 6), clearly demonstrating its protein kinase activity. Autophosphorylation of PKC-L was enhanced by the addition of phosphatidylserine and phorbol esters. Our data also indicated that under the assay conditions used, the kinase activity of

PKC-L was independent of the presence of Ca^{2+}. This is clearly different from the activity of PKC-α, which is regulated by Ca^{2+}. Our attempts to measure the kinase activity of PKC-L expressed in COS cells in PKC assays with histone as a substrate for phosphorylation (11) have not been successful, although PKC-α was active in parallel experiments (data not shown). Moreover, treatment of partially purified PKC-L (on DEAE-Sephacel columns) with antibodies against PKC-α to separate the activity of PKC-L from the endogenous activity of PKC-α present in COS cells did not significantly improve these results. These experiments probably indicate that histone is a poor substrate for PKC-L phosphorylation.

DISCUSSION

In the present study we isolated and characterized cDNA clones coding for PKC-L, a new member of the PKC-related subfamily PKC-δ, PKC-ε, and PKC-ζ (27, 30, 31). PKC-L shares structural features and a similar overall structure with these PKC-related subspecies; most notably, it also lacks the C_2 conserved region, previously implicated in the interaction with Ca^{2+} (30). The absence of a Ca^{2+}-regulatory domain clearly distinguishes PKC-L and other members of this subfamily from classical PKC molecules. However, its specific tissue distribution is unique among members of the PKC-related subfamily or classical PKCs. In contrast to other PKCs, whose expression is enriched in brain tissue, PKC-L is expressed mainly in lung tissue and is found also in skin and heart tissues. Its preferential presence in lung and skin tissues may indicate expression in epithelial cells that are rapidly turned over.

Phorbol esters have been reported to induce a pleotropic response in a wide variety of cell types, including effects on cell morphology, cell-cell communication, membrane transport, phospholipid metabolism, protein phosphorylation, induction of viral and cellular genes, and cell proliferation and differentiation (42, 43). Earlier biochemical studies showed that PKC is the major receptor for phorbol esters (7, 22, 24). Lately, data have accumulated indicating that in fact several distinct PKC molecules may serve as phorbol ester receptors. Each of the four classical PKC molecules has been shown to possess this activity (26). nPKC, from rabbit brain (corresponds to the ε subspecies), a member of the PKC-related subfamily, has also been shown to bind phorbol esters (27). Here we provide evidence that PKC-L also has this capacity and that its direct interaction with phorbol esters can induce its intrinsic kinase activity. Thus, PKC-L may represent another cellular candidate that is able to transmit signals mediated by phorbol esters. Furthermore, we demonstrate that PKC-L is expressed in human epithelial skin-derived cell lines and in the human epidermoid carcinoma line A431 (Fig. 4). Hence, interpretation of experiments involving phorbol ester treatment of these cells as well as skin tissues should also take into account the presence of PKC-L.

The existence of a family of classical PKC and PKC-related molecules, of which more than one subtype is usually expressed in a particular cell type, suggests that distinct PKC isoforms may activate different cellular pathways. This could explain, at least in part, the diversity of responses observed upon PKC activation, especially by phorbol esters. Moreover, the nature of these responses is probably determined by the differential expression of specialized isoenzymes in individual cell types. Thus, expression of different PKC forms may provide additional specificity to the cellular signaling systems, where a great diversity of cellular pathways are thought to be activated by a limited number of signaling molecules such as DAG. It should be noted, however, that although many studies have demonstrated coupling of agonist stimulation to DAG production through phosphoinositide-specific phospholipase C (17), recent evidence also demonstrates the accumulation of unusual DAG species as well as ether-linked diaglycerides, both probably originating from the hydrolysis of choline glycerophospholipids (10, 14). Ether-linked diaglycerides were also shown to activate PKC in vitro (14). The generation of multiple DAG species during agonist stimulation in conjunction with the presence of multiple PKC isoforms raises the interesting possibility that distinct PKC isoforms, probably localized at separate subcellular compartments, respond differently to these second-messenger activator molecules. Therefore, cell-specific responses to an agonist may reflect the specific phospholipase (i.e., A_2, C, or D) or the G protein with which the agonist-receptor associates, the fatty acid and polar headgroup composition of the membrane phospholipids, and the specific isoenzymes of PKC that are expressed in a particular cell. The isolation of cDNAs coding for each of the PKC family members, including PKC-L, enables us to characterize in detail the consequences of their introduction into cells. This will allow us to discern their precise function and biochemical modes of activation and to monitor cellular events occurring after their activation in response to external cell stimulation.

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